



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁵ : A23J 3/34, A23L 1/305	A1	(11) International Publication Number: WO 93/04593 (43) International Publication Date: 18 March 1993 (18.03.93)
(21) International Application Number: PCT/IE92/00006 (22) International Filing Date: 28 August 1992 (28.08.92) (30) Priority data: 3057/91 30 August 1991 (30.08.91) IE 786,111 4 November 1991 (04.11.91) US (71) Applicant (for all designated States except US): TEAGASC, THE AGRICULTURE AND FOOD DEVELOPMENT AUTHORITY [IE/IE]; 19 Sandymount Avenue, Dublin 4 (IE). (72) Inventors; and (75) Inventors/Applicants (for US only) : O'CALLAGHAN, Da- niel, Martin [IE/IE]; Tullylease, Charleville, Co. Cork (IE). DONNELLY, William, John [IE/IE]; Ballyder- own, Killworth, Fermoy, Co. Cork (IE).		(74) Agent: GATES, Marie, Christina, Esther; Tomkins & Co., 5 Dartmouth Road, Dublin 6 (IE). (81) Designated States: AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, US, Euro- pean patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: HYPOALLERGENIC WHEY PROTEIN HYDROLYSATE (57) Abstract <p>A process for the production of a hypoallergenic whey protein hydrolysate involves hydrolysing a whey-based substrate with a proteolytic enzyme, thermally inactivating the enzyme and microfiltering the product of hydrolysis. The hypoallergenic whey protein hydrolysate finds use in the manufacture of infant formulae and special dietetic foodstuffs.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	MN	Mongolia
AU	Australia	FR	France	MR	Mauritania
BB	Barbados	GA	Gabon	MW	Malawi
BE	Belgium	GB	United Kingdom	NL	Netherlands
BF	Burkina Faso	GN	Guinea	NO	Norway
BG	Bulgaria	GR	Greece	NZ	New Zealand
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	PT	Portugal
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland	KR	Republic of Korea	SE	Sweden
CI	Côte d'Ivoire	LJ	Liechtenstein	SK	Slovak Republic
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CS	Czechoslovakia	LU	Luxembourg	SU	Soviet Union
CZ	Czech Republic	MC	Monaco	TD	Chad
DE	Germany	MG	Madagascar	TG	Togo
DK	Denmark	MI	Mali	UA	Ukraine
ES	Spain			US	United States of America

5

Title of the Invention"Hypoallergenic whey protein hydrolysate"

10

Technical Field

The present invention relates to a process for the production of a hypoallergenic whey protein hydrolysate and to the product of such a process. The hypoallergenic whey protein hydrolysate finds use in the manufacture of infant formulae and special dietetic foodstuffs.

Background of the Invention20 Field of the Invention

Infant formulae are manufactured based on the nutritional profile of human milk, where the relative concentrations of casein nitrogen, whey protein, nitrogen and non-protein nitrogen are 35%, 40% and 25% respectively (Packard, 1982). Bovine milk (78% casein, 17% whey protein and 5% NP-N) is the usual substitute for human milk but it requires several modifications. The ratio of casein to whey protein nitrogen for instance must be adjusted to 40:60. This is generally carried out using demineralised whey powder which also advantageously increases the lactose concentration to that of breast milk.

Bovine milk proteins however, are known to give rise to antigenic responses in a small percentage of the population; estimates range from 0.1% to 8% (Clein, 1954, and Collins-Williams, 1962). However, Frier and Kletter (1970) found the incidence to be only about 0.5% when stricter criteria were adopted for the survey. Common allergenic response include diarrhoea, vomiting, intestinal disorders, respiratory problems, dermatitis, irritability, restlessness and loss of appetite

- 2 -

beta-Lactoglobulin (absent in human milk) is the most frequent cause of milk sensitivity. A survey by Saperstein and Anderson (1962) revealed that a variety of infant formulae on the market contained all the major whey protein and casein antigens. Soybean preparations have been used
5 as substitutes for cows milk in baby formulae but can be at least as antigenic as milk protein. (Eastham and Grady, 1978).

Description of the Related Art

- 10 Numerous technological approaches have been taken in attempts to provide non-allergenic/hypoallergenic bovine milk protein for sensitive infants. Ratner *et al* (1958) studied the effect of heat treatment on the allergenicity of whole milk and individual milk proteins. A particular heat denatured milk was shown to have lost the allergenicity
15 of the alpha-lactalbumin fraction and partially lost that of the beta-lactoglobulin fraction. Similar observations were made by others including Kilshaw *et al* (1982) who suggested that a non sensitizing baby milk formula could be produced from heat denatured whey protein. However, they suggested that in order to achieve optimal immunological
20 benefits and nutritional quality, low molecular weight solutes in the whey should be depleted before heat treatment thereby minimising milliard reactions and assuring maximum denaturation of beta-lactoglobulin. Protein insolubility due to heat denaturation is a major disadvantage of such a process. Baldo (1984) demonstrated that
25 even after severe heat treatment (100° for 3 hours) the IgE antibodies in the serum of many patients still reacted with heat-treated proteins and concluded that there was little possibility of producing hypoallergenic milk formulae by heat treatment alone.
- 30 Modification of food proteins by enzymatic hydrolysis is well documented (Adler-Nissen, 1986) and can be used to reduce the allergenicity of bovine milk proteins for inclusion in hypoallergenic baby formulae and special dietetic foods. Hydrolysis of susceptible peptide bonds in native proteins can lead to destruction and unfolding
35 of antigenic determinants (epitopes) on the surface of the protein.

For instance, casein treated with pancreatic protease enzymes was shown to be devoid of antigenicity (Takase *et al* 1977). Pahud *et al*

- 3 -

(1985) and Jost *et al* (1987) produced hypoallergenic whey protein hydrolysates using trypsin. Asselin *et al* (1989) demonstrated that hydrolysis of whey proteins with pepsin followed by alpha-chymotrypsin was the most efficient combination of enzymes to reduce allergenicity of alpha-lactalbumin and beta-Lactoglobulin. Depending on the specificity of the enzyme and rigidity of the three dimensional structure of the protein to be hydrolysed, a preliminary heat treatment may be desirable to make susceptible bonds more accessible to proteolytic degradation. The advantage of enzymic hydrolysis over heat treatment alone is the increased solubility of the product over a broad pH range but sensoric properties may be affected due to increased bitterness.

There are several examples in the patent literature describing the use of enzyme treatment of food proteins and use of ultrafiltration/diafiltration for preparing non-allergenic or hypo-allergenic hydrolystates suitable for inclusion in various dietetic foods, baby formulae and in foods for allergics.

For example, the European Patent Specification No. 0226221.B describes a whey based protein hydrolysate characterised by having a molecular weight profile of not greater than 6,000 Daltons and being free of allergenic substances and lactose. An enzyme hydrolysis process is used to produce the hydrolysate, in which the peptides are harvested from the crude hydrolysate using ultrafiltration (m.w.c.o. 6,000) and/or diafiltration. It is evident from the description of the process that NaOH is added as the sole base to control pH. However, this would lead to excessive levels of Na⁺ in the final product for inclusion in infant formulae. The end product is described as being particularly suitable for allergics and for humans with lactose malabsorption.

European Patent Specification No. 353 122 A discloses a hypoallergenic whey protein hydrolysate of a defined molecular weight profile, i.e. not containing peptides greater than 5,000 Daltons with 35-40% between 1500 and 500 Daltons and a process for producing the same. The process involves enzymatic hydrolysis of whey proteins using an enzyme mixture consisting of chymotrypsin and trypsin with relative activities of from

- 4 -

1.5 to 3.0. The crude hydrolysate is fractionated by ultrafiltration/diafiltration using membranes with a threshold of less than 10,000 Daltons.

5 UK Patent Specification No. 2043651 B describes a process for preparing purified protein hydrolysates, for use in the dietetic field, from animal proteins (meat, fish), vegetable proteins, microbial proteins and milk proteins. The process involves hydrolysing the proteins, heat treating the product of hydrolysis to denature the proteins and then
10 ultrafiltering to remove the denatured proteins. A range of ultrafiltration membranes is described for purifying the crude hydrolysates having cut-off zones of 1,000 to 10,000 Daltons.

PCT Publication No. W087/03785 describes a process in which casein is
15 eliminated from the whey protein material, the casein-free whey protein is hydrolysed with at least one proteinase and the hydrolysate is ultrafiltered through a membrane with a cut-off value of not greater than 20,000 Daltons. A product suitable for use in an hypoallergenic formulae can be produced if a membrane with a cut-off value of 6,000
20 Daltons is used.

European Patent Specification No. 0065663A describes the preparation of a protein hydrolysate for use in an enteric diet in which whey protein is digested with a fungal protease to produce a product in which not
25 more than 25% of the resultant polypeptides contain 10 or more amino-acids.

A process for preparing an hypoallergenic lactoserum hydrolysate is described in European Patent Specification No. 0322589 A, in which
30 lactoserum is subjected to a two step enzymatic hydrolysis. The specification defines "hypoallergenic to mean having no detectable protein of molecular weight greater than 10,000 Daltons".

It is thus generally accepted in the prior art that to produce a
35 hypoallergenic product, the constituent proteins or polypeptides must be smaller than 10,000 Daltons.

In the prior art processes ultrafiltration is used to remove

- 5 -

unhydrolysed protein and aggregated peptides. In these processes the use of ultrafiltration, which excludes peptides of greater than 10,000 Daltons was thought to be necessary to achieve the desired reduction in allergenicity. It has surprisingly been found that microfiltration membranes, which allow the passage of much larger peptides, will also allow the production of a hypoallergenic product, when used in the process of the present invention. Microfiltration offers considerable advantages over ultrafiltration in terms of product yield. In general, microfiltration membranes are less prone to fouling and can be run for longer periods without cleaning.

The crude hydrolysate of this invention consists of both highly aggregated unhydrolysed proteins which are removed by microfiltration, and a mixture of polypeptides ranging in size from 50,000 Daltons to free amino acids which pass through the membrane and are hypoallergenic.

Lactose is present in human milk at about 7% and contributes about 40% of the caloric intake of the milk. Humanised milk/infant formulae are designed to mimic the composition of human milk and therefore lactose must be included. When formulating a hypoallergenic baby formulae the lactose used must be of a quality such that residual protein, remaining in the lactose following the crystallization step in manufacture, is low enough to satisfy the immune-response specification for hypoallergenic formulae. Generally it is impossible to obtain commercial edible and refined lactose to satisfy this specification and thus the production of a hypoallergenic infant formula has been very difficult.

Object of the Invention

It is an object of the present invention to provide a process for the production of an hypoallergenic protein hydrolysate in which yield is increased and cost is reduced. It is a further object to provide such a process in which the level of added salts can be kept low enough to satisfy specifications for inclusion in infant or hypoallergenic formulae. A further object of the invention is to provide a process for producing a hypoallergenic whey protein hydrolysate incorporating lactose.

Summary of the Invention

According to the present invention there is provided a process for the production of an hypoallergenic whey protein hydrolysate comprising
5 hydrolysing a substrate with a proteolytic enzyme, thermally inactivating the enzyme and microfiltering the product of hydrolysis.

The microfiltration step allows the passage of molecules having a range of sizes from free amino-acids to molecules of about 50,000 Daltons.
10 The pore size of the microfiltration membrane may be between 0.02 and 0.3 μm , preferably 0.1 μm .

The substrate may be selected from lactalbumin, whey protein concentrate, demineralized whey powder or a mixture thereof.
15 Optionally, lactose may be added to the substrate before hydrolysis.

The proteolytic enzyme may be a pancreatic, fungal, bacterial or plant protease and may be acidic, neutral or alkaline. The pH and temperature conditions of the process are then suitably adjusted to the
20 optimum requirements of the enzyme.

The invention also provides an hypoallergenic whey protein hydrolysate comprising peptides which range in molecular weight from free amino acids to 50,000 Daltons. The hydrolysate may also comprise lactose.
25

Detailed Description of the Invention

The hypoallergenic whey protein hydrolysate of the present invention is suitable for use in infant formulae. The products of the invention
30 consist of hydrolysed whey protein with a defined molecular weight profile ranging from 50,000 Daltons to free amino acids. An advantage of the present invention is the optional inclusion of lactose which is also subjected to proteolytic treatment to hydrolyse residual proteins present in commercially available lactose, which are known to give rise
35 to antigenic responses. The mineral levels of the products described are maintained within the specifications for infant formulae. The products are 98-100% soluble across a broad pH range and have a very acceptable flavour at neutral pH. Bitterness is not detectable when

- 7 -

the hydrolysate is reconstituted in water at a concentration of 1% protein. Furthermore, the product of the present invention reconstitutes to a clear solution which is very low in colour.

- 5 The process of the invention utilises a range of whey protein substrates, i.e., lactalbumin, whey protein concentrate (WPC), demineralised whey powder, or a mixture thereof. Protein that is not already in a denatured form is given a preliminary heat treatment before addition of the enzyme solution, and before the optional
10 addition of lactose. In addition to the indigenous lactose of the whey protein source, commercial food grade lactose may be added to the hydrolysis mixture before enzyme hydrolysis. In a particular embodiment, the quantity of lactose added is calculated such that the final concentration of lactose and protein in the finished product is
15 70% and 22% respectively. This embodiment is suitable for use as a hypoallergenic baby formulae. Without additional lactose a high protein (80%) hydrolysate is produced instead.

- The preferred enzyme (Pancreatic Trypsin, Novo Industrie A/S, Novo Alle,
20 2880 Bagsvaerd, Denmark) used for the hydrolysis step has been carefully selected from a range of food grade proteases to give the required degree of hydrolysis, molecular weight distribution of polypeptides and to give rise to a non-bitter hydrolysate when used under the conditions described. It is desirable to have a certain
25 proportion (approx 8 - 15%) of the total polypeptide mixture in the region of 50,000 to 5,000 Daltons to provide emulsion stability in the final infant formulae.

- Bitter flavour of protein hydrolysates depends on the degree to which a
30 protein is hydrolysed (% DH) and on the amino acid composition of the peptides produced. Peptides containing a high percentage of hydrophobic amino acids (i.e. Phe, Pro, Val, Trp, Leu, Ile) have been positively correlated with increased bitter flavour. At low degrees of hydrolysis, the hydrophobic amino acids in peptides are unavailable for
35 interaction with the taste buds due to folding and existence of "hairpin" loops within large peptide structures which shield the hydrophobic amino acids. At high degrees of hydrolysis the extent to which hydrophobic interactions and folding of polypeptides occur is

- 8 -

limited and hydrophobic amino acids are 'forced' to exist at the surface of peptides and therefore become available for interaction with taste buds - hence bitter flavour.

- 5 Thus one must choose an enzyme or enzyme mixtures which give rise to peptides with a low average hydrophobicity and also to limit the extent to which the protein is hydrolysed.

This can only be achieved by an extensive empirical screening process
10 and evaluation of hydrolysis conditions. In the present case the pancreatic enzyme preparation from Novo (PTN 3.0S) satisfied the above criteria and was successfully used to achieve a hydrolysate which was judged by a trained taste panel to have a very low bitterness.

- 15 A novel approach has also been adopted for the enzyme hydrolysis step by way of pH control. In the present process a titrant mixture of potassium hydroxide and sodium hydroxide is formulated such that a ratio of 1:3 (w/w) of Na⁺ and K⁺ exists in the finished product. The quantity of titrant used to maintain pH during the enzyme hydrolysis
20 step is calculated such that the final concentration of Na⁺ and K⁺ in the finished product does not exceed the specifications for infant or hypoallergenic formulae; in the case of infant formulae that being 10 mg and 30 mg respectively per gram of pure protein. Once the allowable concentration of Na⁺ and K⁺ has been reached in the
25 hydrolysis mixture the enzyme reaction is allowed to continue without pH control until the required degree of hydrolysis and molecular weight profile has been achieved at which time the enzyme is immediately thermally inactivated.

- 30 The crude hydrolysate is then clarified using a microfiltration membrane of pore size 0.02 to 0.3µm, preferably 0.1µm. Microfiltration, advantageously removes unhydrolysed protein and aggregated peptides from the crude hydrolysate to yield a clear permeate containing a mixture of polypeptides, free amino acids and
35 lactose which can be evaporated and spray dried.

The use of Enzyme Linked Immunosorbent Assay [ELISA] is well described in the literature (Voller, 1976, Ishikawa et al 1981, Wisdom 1981). In

- 9 -

this instance a sandwich ELISA technique was used to determine the level of whey protein antigens remaining in protein hydrolysates, after processing. In principle the sandwich technique involves binding anti-whey antibodies to the ELISA plate and reacting the bound material
5 with protein hydrolysate. This is followed by incubation with anti-whey antibodies conjugated to the enzyme HRP. Enzyme substrate is then added and the amount of colour developed by enzyme-catalysed conversion of substrate is a measure of the amount of antigenic material in the protein hydrolysate.

10

Results from the ELISA reactivity experiments against a total whey protein antisera showed that the antigenicity of the whey protein hydrolysates produced according to the present invention was reduced by at least 4 orders of magnitude and in most cases by at least 5 orders
15 of magnitude when compared with a standard whey protein concentrate.

EXAMPLE 1.

A slurry of lactalbumin (Alatal 560, New Zealand) was prepared by
20 adding 18.75 kg Alatal (86% protein) to 175 litres of pasteurised water in a 210 litre batch stirred tank reactor (BSTR). The temperature of the slurry was adjusted to 50°C and the pH increased to 8.0 with automatic addition of a titrant mixture consisting of 2.56 M KOH and 1.44 M NaOH (3:1 w/w K⁺/Na⁺), using an industrial pH stat. The
25 total quantity of titrant mixture added to the hydrolysis reaction was calculated so that the final concentrations of Na⁺ and K⁺ in the crude hydrolysate mixture did not exceed 7mg and 21mg per gram of pure protein respectively. At a protein permeation rate of approximately 70% through the microfiltration membrane this should yield a final
30 product with less than 10 mg and 30 mg of Na⁺ and K⁺ respectively per gram of pure protein.

The volume of the reaction mixture was made up to 195 litres with deionised water. The proteolytic enzyme mixture was added once the
35 lactalbumin slurry had equilibrated at 50°C and pH 8.0 for a minimum of 30 minutes. The enzyme [320 g of food grade Trypsin (PTN 3.0S, Novo)] was dissolved in 5 litres of deionised water before addition to the reaction mixture. In this instance the enzyme to substrate ratio

- 10 -

(E/S) was equivalent to 2%. The pH stat was immediately activated and the remaining KOH/NaOH mixture continuously added to maintain pH at 8.0. When the allowable concentration of K^+ and Na^+ was reached in the crude hydrolysate, the enzyme reaction was continued without pH control. The reaction was stopped (by thermal inactivation of the enzyme) when the required DH (Degree of Hydrolysis) and molecular weight profile had been achieved which typically took 5-6 hours. Thermal inactivation was achieved by increasing the temperature of the crude hydrolysate to 80°C and maintaining this temperature for 20 minutes. The hydrolysate was then chilled to 4°C and held overnight for further processing.

Membrane Processing

The temperature of the crude hydrolysate was adjusted to 50°C and transferred to the balance tank of the microfiltration unit. In this example, the microfiltration module consisted of an Abcor hollow fibre tangential flow membrane, 5 m², and with a nominal pore size of 0.1 µm (Koch International).

Microfiltration was continued in batch mode with the retentate recycled to the feed tank, the permeate was collected in 25 litre plastic containers, weighed and pooled to form a bulk permeate. 150 litres of permeate was collected, which represents a volume concentration reduction (VCR) of 4. The pooled permeate was heated to 75°C for 15 mins (which resulted in less foaming during the evaporation step), evaporated to 40% T.S., and spray dried to yield a 'clarified' lactalbumin hydrolysate powder. The physico-chemical properties of the powder are outlined in Table 1, and the molecular weight profile is shown in Table 2. The hydrolysate satisfied the criteria for hypoallergenic baby formulae. The antigenicity of the whey protein hydrolysate was reduced by approximately 5 orders of magnitude compared with a standard whey protein concentrate not subjected to any modifications.

35

- 11 -

TABLE 1: Product profile of a spray dried hypoallergenic whey protein hydrolysate powder prepared from Lactalbumin.

5	<u>Component</u>	<u>Concentration</u>
	Protein	82.68%
10	Ash	7.15%
	Fat	< 0.2%
	Moisture	3.15%
	Lactose	2.28%
	Na ⁺	9.68 mg/g (pure protein)
15	K ⁺	25.74 mg/g (pure protein)
	pH (10% soln.)	7.16

20

TABLE 2: Molecular weight profile of a hypoallergenic whey protein hydrolysate powder prepared from Lactalbumin.

25	<u>Molecular weight (Daltons)</u>	<u>% of total</u>
	>50,000	0
	50,000 - 5,000	11.2
	5,000 - 1,500	33.3
30	1,500 - 1,000	21.8
	1,000 - 100	31.6
	<100	2

35 EXAMPLE 2

Essentially the same process as in Example 1 was used except that lactose was added to the reaction mixture before enzyme addition.

- 12 -

Additional lactose was added to give a final ratio of 2.2:1 lactose to protein in the crude hydrolysate mixture. This was calculated taking into account the relative permeation rates of the protein and lactose through the microfiltration membrane, i.e. 70% and 95% are typical permeation rates for protein and lactose respectively when using the 0.1um Abcor spirally wound membrane. The aim was to have a protein content in the finished product of 22% and 70% lactose.

Lactose is added to the hydrolysis mixture before enzyme addition so that any residual protein remaining from the crystallisation process during the manufacture of lactose is hydrolysed along with the lactalbumin proteins. A slurry of lactalbumin (Alatal 560, New Zealand) was prepared by adding 18.75 kg Alatal (86% protein) to 150 litres of pasteurised water in a 210 litre batch stirred tank reactor (BSTR). The temperature of the slurry was adjusted to 50°C. Commercial food grade lactose (35.45 kg) was then added to the reaction mixture and allowed to dissolve thoroughly. The pH of the mixture was increased to 8.0 with automatic addition of a titrant mixture consisting of 2.56 M KOH and 1.44 NaOH (3:1 w/w, K⁺/Na⁺), using an industrial pH stat. The total quantity of titrant mixture added to the hydrolysis reaction was calculated so that the final concentrations of Na⁺ and K⁺ in the crude hydrolysate mixture did not exceed 7 mg and 21 mg per gram of pure protein respectively. At a protein permeation rate of approximately 70% through the microfiltration membrane this should yield a final product with less than 10 mg and 30 mg of Na⁺ and K⁺ respectively per gram of pure protein.

The volume of the reaction mixture was made up to 195 litres with deionised water. The proteolytic enzyme mixture was added once the lactalbumin slurry had equilibrated at 50°C and pH 8.0 for a minimum of 30 minutes. The enzyme [320g of food grade Trypsin (PTN 3.0S, Novo)] was dissolved in 5 litres of deionised water before addition to the reaction mixture. In this instance the enzyme to substrate ratio (E/S) was equivalent to 2%. The pH stat was immediately activated and the remaining KOH/NaOH mixture continuously added to maintain pH at 8.0. When the allowable concentration of K⁺ and Na⁺ was reached in the crude hydrolysate, the enzyme reaction was continued without pH control. The reaction was stopped (by thermal inactivation of the

- 13 -

enzyme) when the required DH and molecular weight profile had been achieved, which typically took 5-6 hours. Thermal inactivation was achieved by increasing the temperature of the crude hydrolysate to 80°C and maintaining this temperature for 20 minutes. The

5 hydrolysate was then chilled to 4°C and held overnight for further processing.

Membrane Processing

10 The temperature of the crude hydrolysate was adjusted to 50°C and transferred to the balance tank of the microfiltration unit. In this example, the microfiltration module consisted of an Abcor hollow fibre tangential flow membrane, 5m², and with a nominal pore size of 0.1 µm (Koch International).

15 Microfiltration was continued in batch mode with the retentate recycled to the feed tank, the permeate was collected in 25 litre plastic containers, weighed and pooled to form a bulk permeate. 150 litres of permeate was collected, which represents a volume concentration

20 reduction (VCR) of 4. The pooled permeate was heated to 75°C for 15 mins, evaporated to 40% T.S., and spray dried to yield a 'clarified' lactalbumin hydrolysate powder. The physico-chemical properties of the powder are outlined in Table 3, and the molecular weight profile is shown in Table 4. The hydrolysate satisfied the criteria for

25 hypoallergenic baby formulae. The antigenicity of the whey protein hydrolysate was reduced by approximately 5 orders of magnitude compared with a standard whey protein concentrate not subjected to any modifications.

30

35

- 14 -

Table 3: Product profile of a spray dried hypoallergenic whey protein hydrolysate powder prepared from lactalbumin with added lactose.

5

	<u>Component</u>	<u>Concentration</u>
10	Protein	20.75%
	Ash	2.12%
	Fat	< 0.2%
	Moisture	2.51%
	Lactose	69.5%
15	Na ⁺	9.43 mg/g (pure protein)
	K ⁺	27.79 mg/g (pure protein)
	pH(10% soln.)	6.42

20

Table 4: Molecular weight profile of a hypoallergenic whey protein hydrolysate powder prepared from lactalbumin with added lactose.

25

	<u>Molecular weight (Daltons)</u>	<u>% of total</u>
	>50,000	0
	50,000 - 5,000	2.3
30	5,000 - 1,500	21.8
	1,500 - 1,000	10.7
	1,000 - 100	57.9
	<100	7.3

35

- 15 -

Example 3

A solution of whey protein concentrate (WPC 80, Milei, West Germany) was prepared by adding 20 kg WPC 80 (80% protein) to 150 litres of deionised water in a 210 litre batch stirred tank reactor (BSTR) at 50°C with the aid of a Silverson mixer. The concentrated WPC solution was allowed to dissolve completely with continuous stirring at 50°C for 2 hours. The solution of WPC was then heat denatured by increasing the temperature to 95°C and maintaining this temperature for 30 minutes. The mixture was stirred vigorously during the heat treatment to prevent clumping of aggregates. After the heat treatment the temperature of the WPC slurry was reduced to 50°C. The pH of the mixture was increased to pH 8.0 with automatic addition of a titrant mixture consisting of 2.56 M KOH and 1.44 M NaOH (3:1 w/w, K⁺/Na⁺), using an industrial pH stat. The total quantity of titrant mixture added to the hydrolysis reaction was calculated so that the final concentration of Na⁺ and K⁺ in the crude hydrolysate mixture did not exceed 7 mg and 21 mg per gram of pure protein respectively. At a protein permeation rate of approximately 70% through the microfiltration membrane this should yield a final product with less than 10 mg and 30 mg of Na⁺ and K⁺ respectively per gram of pure protein.

The volume of the reaction mixture was made up to 195 litres with deionised water. The proteolytic enzyme mixture was added once the heat denatured WPC slurry had equilibrated at 50°C and pH 8.0 for a minimum of 30 minutes. The enzyme [320 g of food grade trypsin (PTN 3.0S, Novo)] was dissolved in 5 litres of deionised water before addition to the reaction mixture. In this instance the enzyme to substrate ratio (E/S) was equivalent to 2%. The pH stat was immediately activated and the remaining KOH/NaOH mixture continuously added to maintain pH at 8.0. When the allowable concentration of K⁺ and Na⁺ was reached in the crude hydrolysate, the enzyme reaction was continued without pH control. The reaction was stopped (by thermal inactivation) when the required DH and molecular weight profile had been achieved, which typically took 5-6 hours. Thermal inactivation was achieved by increasing the temperature of the crude hydrolysate to 80°C and maintaining this temperature for 20 minutes. The

- 16 -

hydrolysate was then chilled to 4°C held overnight for further processing.

Membrane Processing

5

The temperature of the crude hydrolysate was adjusted to 50°C and transferred to the balance tank of the microfiltration unit. In this example, the microfiltration module consisted of an Abcor hollow fibre tangential flow membrane, 5m², and with a nominal molecular weight cutoff of 0.1 µm (Koch International).

10

Microfiltration was continued in batch mode with the retentate recycled to the feed tank, the permeate was collected in 25 litre plastic containers, weighed and pooled to form a bulk permeate. 150 litres of permeate was collected, which represents a volume concentration reduction (VCR) of 4. The pooled permeate was heated to 75°C for 15 mins, evaporated to 40% T.S., and spray dried to yield a 'clarified' whey protein hydrolysate powder. The physico-chemical properties of the powder are outlined in Table 5, and the molecular weight profile is shown in Table 6. The hydrolysate satisfied the criteria for hypoallergenic baby formulae. The antigenicity of the whey protein hydrolysate was reduced by approximately 5 orders of magnitude compared with a standard whey protein concentrate not subjected to any modifications.

25

30

35

- 17 -

Table 5: Product profile of a spray dried hypoallergenic whey protein hydrolysate powder prepared from WPC.

5	<u>Component</u>	<u>Concentration</u>
	Protein	77.15%
	Ash	7.26%
10	Fat	0.2%
	Moisture	2.30%
	Lactose	4.83%
	Na ⁺	8.14 mg/g (pure protein)
	K ⁺	21.92 mg/g (pure protein)
15	pH(10% soln.)	6.97

Table 6: Molecular weight profile of a hypoallergenic whey protein hydrolysate powder prepared from WPC.

25	<u>Molecular weight (Daltons)</u>	<u>%of total</u>
	>50,000	0
	50,000 - 5,000	4.9
	5,000 - 1,500	24
	1,500 - 1,000	11
30	1,000 - 100	53.9
	<100	5.6

Example 4

35

Essentially the same process as in Example 3 was used except that lactose was added to the reaction mixture after the heat denaturation step. Additional lactose was added to give a final ratio of 2.2:1

- 18 -

lactose: protein in the crude hydrolysate mixture. This was calculated taking into account, the permeation rates of the protein and lactose through the microfiltration membrane, i.e. 70% and 95% are typical permeation rates for protein and lactose respectively when using the 0.1um Abcor spiral membrane. It was aimed to have a protein content in the finished product of 22% protein and 70% lactose.

Lactose is added to the hydrolysis mixture before enzyme addition so that any residual protein remaining from the crystallisation process during the manufacture of lactose is hydrolysed along with the heat denatured WPC.

A solution of whey protein concentrate (WPC 80, Mieli West Germany) was prepared by adding 20 kg WPC 80 (80% protein) to 150 litres water in a 210 litre batch stirred tank reactor (BSTR) at 50°C with the aid of a Silverson mixer. The concentrated WPC solution was allowed to dissolve completely with continuous stirring at 50°C for 2 hours. The solution of WPC was then heat denatured by increasing the temperature to 95°C and maintaining this temperature for 30 minutes. The mixture was stirred vigorously during the heat treatment to prevent clumping of aggregates. After the heat treatment the temperature of the WPC slurry was reduced to 50°C. Commercial food grade lactose (35.45 kg) was then added to the reaction mixture and allowed to dissolve thoroughly. The pH of the mixture was increased to pH 8.0 with automatic addition of a titrant mixture consisting of 2.56 M KOH and 1.44 M NaOH (3:1 w/w, K^+/Na^+), using an industrial pH stat. The total quantity of titrant mixture added to the hydrolysis reaction was calculated so that the final concentrations of Na^+ and K^+ in the crude hydrolysate mixture did not exceed 7 mg and 21 mg per gram of pure protein respectively. At a protein permeation rate of approximately 70% through the microfiltration membrane this should yield a final product with less than 10 mg and 30 mg for Na^+ and K^+ respectively per gram of pure protein.

The volume of the reaction mixture was made up to 195 litres with deionised water. The proteolytic enzyme mixture was added once the slurry of heat-denatured WPC/lactose had equilibrated at 50°C and pH 8.0 for a minimum of 30 minutes. The enzyme [320 g of food grade

- 19 -

trypsin (PTN 3.0S, Novo)] was dissolved in 5 litres of deionised water before addition to the reaction mixture. In this instance the enzyme to substrate ratio (E/S) was equivalent to 2%. The pH stat was immediately activated and the remaining KOH/NaOH mixture continuously added to maintain pH at 8.0. When the allowable concentration of K^+ and Na^+ was reached in the crude hydrolysate, the enzyme reaction was continued without pH control. The reaction was stopped by thermal inactivation of the enzyme when the required DH and molecular weight profile had been achieved, which typically took 5-6 hours. Thermal inactivation was achieved by increasing the temperature of the crude hydrolysate to 80°C and maintaining this temperature for 20 minutes. The hydrolysate was then chilled to 4°C and held overnight for further processing.

15 Membrane Processing

The temperature of the crude hydrolysate was adjusted to 50°C and transferred to the balance tank of the microfiltration unit. In this example, the microfiltration module consisted of an Abcor hollow fibre tangential flow membrane, 5m², and with a nominal pore size of 0.1 µm (Koch International).

Microfiltration was continued in batch mode with the retentate recycled to the feed tank, the permeate was collected in 25 litre plastic containers, weighed and pooled to form a bulk permeate. 150 litres of permeate was collected, which represents a volume concentration reduction (VCR) of 4. The pooled permeate was heated to 75°C for 15 mins, evaporated to 40% T.S., and spray dried to yield a 'clarified' whey protein hydrolysate powder. The physico-chemical properties of the powder are outlined in Table 7, and the molecular weight profile is shown in Table 8. The hydrolysate satisfied the criteria for hypoallergenic baby formulae. The antigenicity of the whey protein hydrolysate was reduced by approximately 5 orders of magnitude compared with a standard whey protein concentrate not subjected to any modifications.

- 20 -

Table 7: Product profile of a spray dried hypoallergenic whey protein hydrolysate powder prepared from WPC with added lactose.

5		
	<u>Component</u>	<u>Concentration</u>
	Protein	20.83%
10	Ash	2.10%
	Fat	< 0.2%
	Moisture	1.73%
	Lactose	68.7%
	Na ⁺	8.0 mg/g (pure protein)
15	K ⁺	27.27mg/g (pure protein)
	pH (10% soln)	6.97

20

Table 8: Molecular weight profile of a hypoallergenic whey protein hydrolysate powder prepared from WPC with added lactose.

25	<u>Molecular weight (Daltons)</u>	<u>% of total</u>
	>50,000	0
	50,000 - 5,000	4.8
	5,000 - 1,500	23.8
30	1,500 - 1,000	10.6
	1,000 - 100	54.5
	<100	6.3

35 Example 5

Essentially the same process as in Example 4 was used except that an enzyme/substrate ratio of 0.5% was used, and the hydrolysis reaction

- 21 -

was continued for 16 hours. The enzyme [80g food grade trypsin (PTN 3.05, Novo)] was dissolved in 5 litres of deionised water before addition to the reaction mixture. Apart from the lower enzyme/substrate ratio and increased hydrolysis time, all other process variables were identical to Example 4.

The physico-chemical properties of the powder are outlined in Table 9 and the molecular weight profile is shown in Table 10. The hydrolysate satisfied the criteria for hypoallergenic baby formulae. The antigenicity of the whey protein hydrolysate was reduced by approximately 5 orders of magnitude compared with a standard whey protein concentrate not subjected to any modifications.

In all the described examples, the concentration of enzyme is optional. Where a lower concentration is used, a longer reaction time is necessary to achieve the desired degree of hydrolysis, and vice versa.

Table 9: Product profile of a spray dried hypoallergenic whey protein hydrolysate powder prepared from WPC with added lactose.

<u>Component</u>	<u>Concentration</u>
25 Protein	20.07%
Ash	1.79%
Fat	< 0.2%
Moisture	4.0%
Lactose	70.96%
30 Na ⁺	9.80 mg/g (Pure Protein)
K ⁺	28.0 mg/g (Pure Protein)
pH (10% soln)	6.9

35

- 22 -

Table 10: Molecular weight profile of a hypoallergenic whey protein hydrolysate powder prepared from WPC with added lactose.

	<u>Molecular weight (Daltons)</u>	<u>% of total</u>
5	>50,000	.04
	50,000 - 5,000	4.99
	5,000 - 1,500	26.16
	1,500 - 1,000	14.99
10	1,000 - 100	50.28
	<100	3.55

Because the hypoallergenic whey protein hydrolysate of the present invention contains molecules of molecular weight up to 50,000 daltons, a lower degree of hydrolysis is required in the manufacturing process than is required to produce previously known hypoallergenic hydrolysates.

This lower degree of hydrolysis means that less salts have to be added to the product for neutralization. Additionally, the more hydrolysed a product is, the more difficult it is to achieve emulsion stability when utilizing the product, and stabilising agents must be used. Obviously, it is desirable, when producing a food product, to minimise the amount of stabilising agents or added salts which must be added to the product. The product and process of the present invention thus offer considerable advantages over the prior art products and processes.

A feature of the hydrolysates produce by this invention is the excellent flavour of the hydrolysate. A panel of trained tastes judged the product to have very low bitterness or other off-flavour.

The level of bitterness was assessed by a scaling method. A taste panel was presented with two standards representing the extremes of bitterness to be tasted. These were water and a solution of caffeine (0.03%) and were allocated a score of 0 and 10 respectively. Various reference samples of different bitterness were then presented and the panel was asked to allocate a score from 0 to 10 to them. When the

- 23 -

panel were confident in their ability to rank the reference samples consistently, they were presented with the test hydrolysate samples (1% solution in water) and asked to allocate them a score in the same way. The samples consistently scored 2 or less, and on that basis were
5 adjudged to have a very low level of bitterness.

A further beneficial property is that the product forms a solution which is visually clear.

10

15

20

25

30

35

REFERENCES

- Asselin, J., Herbert, J., and Amiot, J. (1989). Effect of in vitro proteolysis on the allergenicity of major whey proteins. J. Food Sci., 54, 1037.
- Adler-Nissen, J. (1986). Enzyme Hydrolysis of Food Proteins. Elsevier Applied Science.
- 10 Baldo, B.A. (1984). Milk allergies. Aust. J. Dairy Technol. Sept. 120.
- Clein, N.W. (1954). Cow's milk allergy in infants. Ped. Clin. N. Amer., 4, 949.
- 15 Collins-Williams, C. (1962). Cow's milk allergy in infants and children. Int. Arch Allerg., 20, 38.
- Eastham, E.J. and Grady, M.I. (1978). Antigenicity of infant formulas: Role of immature intestine on protein permeability. J. Paediatrics, 93, 561.
- 20 Frier, S., and Kletter, B. (1970). Milk allergy in infants and young children. Clin Ped., 9, 449.
- 25 Ishikawa, E., Kawai, T., Miyai, K. (1981). Enzyme Immunoassay Igaku-Shoin Tokyo - New York.
- Jost, R., Monti, J.C. and Pahud, J.J. (1987). Whey protein Allergenicity and its reduction by technological means. Food Technol., 41, (10), 118.
- 30 Kilshaw, P.J., Heppel, L.M.J. and Ford, J.E. (1982). Effects of heat treatment of cow's milk and whey on the nutritional quality and antigenic properties. Arch Dis Child, 57, 842.
- 35 Packard, V.S. (1982). Human Milk and Infant Formula. Academic Press, New York.

- 25 -

Pahud, J.J., Monti, J.C., and Jost, R. (1985). Allergenicity of whey proteins: Its modification by tryptic in-vitro hydrolysis of the protein. J. Pediatr. Gastro. and Nutr. 4, 408.

- 5 Ratner, B. Dworetzky, M., Satoko, O. and Aschheim, L. (1958). Studies on the allergenicity of cow's Milk, 2: Effect of heat treatment on the allergenicity of milk and protein fractions from milk as tested in guinea pigs by parenteral sensitization and challenge. Paediatrics. 22, 648.

10

Saperstein, S. and Anderson, D.W. (1962). Antigenicity of milk proteins of prepared formulas measured by precipitin ring test and passive cutaneous anaphylaxis in the guinea pig. J. Paediatrics, 61, 196.

15

Takase, M., Fukuwatari, Y., Kawase, K., et al. (1977). Antigenicity of casein enzymatic hydrolysates. J.Dy.Sci. 62, 1570.

- 20 Voller, A., Bidwell, D.E. and Bartlett, A. (1976). Enzyme Immunoassays in Diagnostic Medicine. Bull W.H.O. 53 55-63.

Wisdom, B.G. (1981). Recent progress in the Development of Enzyme Immunoassay. The Ligand Review. 3, 44-49.

25

30

35

CLAIMS

1. A process for the production of a hypoallergenic whey protein hydrolysate comprising hydrolysing a substrate with a proteolytic
5 enzyme, thermally inactivating the enzyme and microfiltering the product of hydrolysis.
2. A process as claimed in claim 1 wherein the microfiltration step allows the passage of molecules having a range of sizes from about
10 50,000 Daltons to free amino-acids.
3. A process as claimed in claim 2 wherein a microfiltration membrane having a pore size of between 0.02 and 0.3 μ m is used.
- 15 4. A process as claimed in claim 3 wherein the membrane pore size is 0.1 μ m.
5. A process as claimed in any preceding claim wherein the substrate is chosen from lactalbumin, whey protein concentrate,
20 demineralized whey powder or a mixture thereof.
6. A process as claimed in any preceding claim wherein lactose is added to the substrate before hydrolysis by the proteolytic enzyme.
- 25 7. A process as claimed in any preceding claim wherein the substrate is subjected to a preliminary heat treatment to denature protein.
8. A process as claimed in claim 7 wherein the substrate is heated
30 to at least 50⁰C at pH 8 for at least 30 minutes.
9. A process as claimed in any preceding claim wherein the proteolytic enzyme is chosen from pancreatic, fungal, bacterial or plant proteases.
- 35 10. A process as claimed in claim 9 wherein the protease is pancreatic trypsin.

- 27 -

11. A process as claimed in claim 10 wherein hydrolysis is carried out at pH 8 at 50°C.
12. A process as claimed in any preceding claim wherein the pH is maintained by the addition of a mixture of potassium hydroxide and sodium hydroxide.
13. A process as claimed in claim 12 wherein the ratio of $\text{Na}^+:\text{K}^+$ in the mixture is 3:1 (w/w).
14. A process as claimed in any preceding claim in which thermal inactivation of the proteolytic enzyme takes place at a temperature of at least 70°C for at least 20 minutes.
15. A process as claimed in any preceding claim wherein the antigenicity of the whey protein hydrolysate is reduced by approximately 5 orders of magnitude compared with a standard whey protein concentrate.
16. A hypoallergenic whey protein hydrolysate comprising peptides with molecular weight of up to about 50,000 Daltons.
17. A hypoallergenic whey protein hydrolysate as claimed in claim 16 also containing lactose.
18. A hypoallergenic whey protein hydrolysate which is visually clear and has a non-bitter taste comprising peptides with molecular weight of up to about 50,000 Daltons.
19. A foodstuff comprising a hydrolysate as claimed in any of claims 16 to 18.
20. A foodstuff composition for infants containing a hypoallergenic whey protein hydrolysate comprising peptides with molecular weights of up to about 50,000 and having a protein content of between 22% and 80%, with or without added lactose.
21. A hypoallergenic whey protein hydrolysate comprising 77.15%

- 28 -

protein, 7.26% ash, 0.2% fat, 2.30% moisture, 4.83% lactose, 8.14mg/g (pure protein) Na^+ , 21.92mg/g (pure protein) K^+ and having a pH of 6.97 in a 10% (w/v) solution.

- 5 22. A hypoallergenic whey protein hydrolysate comprising 20.83% protein, 2.10% ash, less than 0.2% fat, 1.73% moisture, 68.7% lactose, 8.0mg/g (pure protein) Na^+ , 27.27mg/g (pure protein) K^+ and having a pH of 6.97 in a 10% (w/v) solution.
- 10 23. A hypoallergenic whey protein hydrolysate having an antigenicity reduced by approximately 5 orders of magnitude compared with a standard whey protein concentrate.
- 15 24. A process for the production of a hypoallergenic whey protein hydrolysate substantially as described herein with reference to the Examples.
- 20 25. A hypoallergenic whey protein hydrolysate whenever prepared by a method as claimed in any of Claims 1 to 15 or 25.
26. A hypoallergenic whey protein hydrolysate substantially as described herein with reference to the Examples.
- 25 27. Use of an hypoallergenic whey protein hydrolysate as claimed in any of claims 16 to 18, 21 to 23, 25, or 26 in the manufacture of a hypoallergenic infant milk formulation.

30

35

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IE 92/00006

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A23J3/34; A23L1/305

II. FIELDS SEARCHEDMinimum Documentation Searched⁷

Classification System

Classification Symbols

Int.Cl. 5

A23J ;

A23L ;

A23C

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸**III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹**

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	EP,A,0 353 122 (UNION DES COOPERATIVES LAITIERS D'ISIGNY-SUR-MER) 31 January 1990 cited in the application	1,5, 9-12,14
Y	see column-3, line 22 - line 31 see column 6, line 56 - line 61 see column 9, line 2 - line 18	2-4,7
Y	EP,A,0 421 309 (SANDOZ NUTRITION) 10 April 1991 see claim 22 see page 1, line 49 - page 2, line 39	7
	-/-	

¹⁰ Special categories of cited documents:¹⁰ "A" document defining the general state of the art which is not considered to be of particular relevance¹⁰ "E" earlier document but published on or after the international filing date¹⁰ "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)¹⁰ "O" document referring to an oral disclosure, use, exhibition or other means¹⁰ "P" document published prior to the international filing date but later than the priority date claimed¹¹ "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention¹¹ "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step¹¹ "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.¹¹ "A" document member of the same patent family**IV. CERTIFICATION**

Date of the Actual Completion of the International Search

04 NOVEMBER 1992

Date of Mailing of this International Search Report

20. 11. 92

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

VUILLAMY V.N.L.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category ^a	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	<p>DATABASE WPIL Section Ch, Week 9023, 1990 Derwent Publications Ltd., London, GB; Class D, AN 90-174649 & JP,A,2 113 859 (NITTO DENKO CORP.) 26 April 1990 see abstract</p>	3,4
Y	<p>EP,A,0 022 019 (INRA) 7 January 1981 see page 19, line 27 - page 20, line 5 see page 21, line 12 - line 24</p>	2
X	<p>EP,A,0 357 776 (TERUMO KABUSHIKI KAISHA) 14 March 1990 see example 3</p>	16,23
P,X	<p>Food Science & Technology Abstracts, 1992. Acc. No. 92-11-p0149, International Food Information Service; NAKAMURA : "Antigenicity of Whey Protein Hydrolysates Fractionated with Ultrafiltration Membrane" & JOURNAL OF JAPANESE SOCIETY OF FOOD SCIENCE AND TECHNOLOGY NIPPON SHOKUHIN KOGYO GAKKAISHI, JAPAN vol. 39, no. 1, 1992, page 113-116 see Abstract</p>	1,5,9,10

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. IE 9200006
SA 63738**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 04/11/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0353122	31-01-90	FR-A- 2634104	19-01-90
		JP-A- 2182155	16-07-90
		US-A- 4981704	01-01-91
EP-A-0421309	10-04-91	JP-A- 3187348	15-08-91
EP-A-0022019	07-01-81	FR-A- 2459620	16-01-81
		AU-B- 535601	29-03-84
		AU-A- 5966780	08-01-81
		CA-A- 1150564	26-07-83
		JP-A- 56032488	01-04-81
		JP-B- 62061039	18-12-87
		US-A- 4427658	24-01-84
EP-A-0357776	14-03-90	WO-A- 8808853	17-11-88